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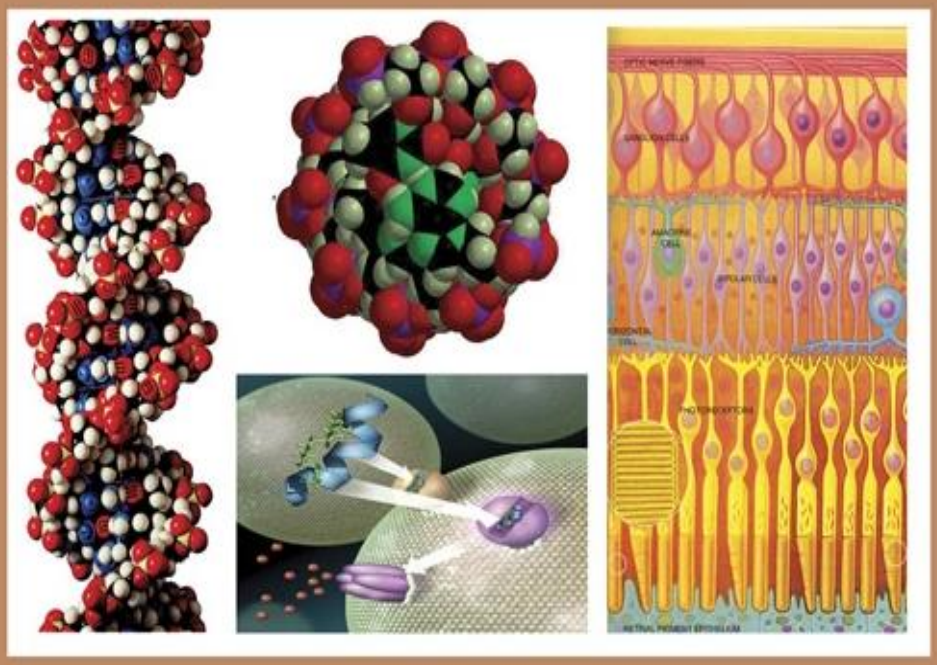

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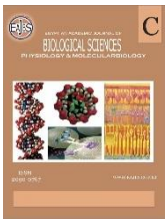

Detection of Biofilm Production and Antibiotic Resistance Pattern In Clinical Isolates from Indwelling Medical Devices

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ABSTRACT

Microbial biofilms pose a great threat for patients requiring indwelling medical devices (IMDs), as it is difficult to remove them. It is, therefore, crucial to follow an appropriate method for the detection of biofilms. The present study focuses on the detection of biofilm formation among the isolates from IMDs. We also aimed to explore the antibiogram of biofilm producers. This prospective analysis included 104 prosthetic samples. After isolation and identification of bacteria following standard methodology, an antibiogram of the isolates was produced following the disc diffusion method. Detection of biofilms was performed by tissue culture plate (TCP), tube adherence (TA) and Congo red agar methods. Over a period of study, 104 IMDs were removed from 78 patients with implantable devices infections. Eighty bacterial strains were isolated and identified, with coagulase-negative staphylococci being the predominant bacteria with 57.5% (46/80). There was a positive correlation between biofilm production and antimicrobial resistance, with the -strongest biofilm producers resistant to more than one antibiotic. For the detection of biofilm production, TA method can be an economical and effective alternative to TCP method. A greater understanding of biofilm detection in pathogen bacteria will help in the development of newer and more effective treatments. The detection of biofilm formation and antibiotic susceptibility patterns helps in choosing the correct antibiotic therapy.

INTRODUCTION

Biofilm is an organized aggregate of microorganisms living within an extracellular polymeric matrix, that they produce and are irreversibly attached to fetish or living surface, which will not remove unless rinsed quickly (Hurlow *et al.*,2015). Compared to their planktonic counterparts, biofilm-associated bacteria exhibit greater resistance to antibiotic agents (Gebreyohannes *et al.*,2019). This increased antibiotic resistance, is mainly due to the limited diffusion of drugs through the biofilm matrix and to physiological changes in bacteria, due to the environmental conditions featuring the biofilm (Franci *et al.*,2018).

Within a biofilm, bacteria communicate with each other, by the production of chemotactic particles or pheromones, a phenomenon called quorum sensing (Danhorn and Fuqua,2007). Biofilm-associated infections on implantable medical devices caused by pathogenic strains, which have negative impacts on public health and medicine, are a major concern (Lindsay and Von Holy,2006). Human diseases in which biofilms have been implicated include urinary tract infections, middle ear infections, formation of dental plaque, gingivitis and less common but more lethal processes such as prosthetic valve endocarditis and cystic fibrosis (Bauer *et al.*,2002). Devices like central venous catheters develop extraluminal biofilms within a week of catheterization a major cause of catheter-associated bloodstream infections (Donlan,2001). In the case of urinary catheters, the risk of catheter-associated infection increases by 10% each day when a catheter is in place (Donlan,2008). Biofilms grow very easily and very rapidly (within 24 h) on endotracheal tubes (ETTs), representing a major cause of ventilator-associated pneumonia (Donlan,2001). The major concern about biofilm infections is the difficulty in their eradication as interior cells in a biofilm are shielded from the immune response of the host as well as from the effect of antibiotic agents (Mekni *et al.*,2012). Meanwhile, in the case of mixed bacterial growth, bacteria once deemed antibiotic sensitive can turn resistant on subsequent antibiotic susceptibility tests due to the horizontal transmission of plasmid-associated drug-resistant genes from resistant bacteria to sensitive bacteria when they become associated within a biofilm (Subramanian *et al.*,2012). Therefore, the present study aimed to detect the presence of biofilm-forming isolates from different medical devices (MDs) and to explore their antibiotic resistance pattern. Knowledge of the main MD strains and related antibiotics susceptibility profile is essential to allow the optimal choice of antibiotic therapy for device-related infections (DRIs).

MATERIALS AND METHODS

MDs Culture and Isolate Identifications*:*

This prospective study was conducted in the Bacteriology Laboratory of Mohamed Boudiaf University Hospital Center, Ouargla, Algeria, from December 2020 to May 2021. IMDs which included central venous femoral (CVF) tip, central venous Jugular (CVJ) tip, Peripheral venous (PV) tip, urinary catheters (UC) tip and endotracheal tube (ET), received in the bacteriology laboratory for culture, and sensitivity was included in the study. They were inoculated into blood agar and Mac Conkey agar (chocolate agar was also used for ET tube) by roll plate method and incubated at 37°C for 24 h.

The isolates were identified by colony morphology, Gram staining and the biochemical identification of the bacteria isolates was performed using the API 20 E, API 20NE and Staph API (Bio-Mérieux, France).

Antibiotic Susceptibility Testing:

Antimicrobial susceptibilities were tested by the disc diffusion method on Mueller-Hinton agar using commercial discs (Bio-Rad Laboratories, France), and interpreted according to the EUCAST guidelines 2021 (EUCAST,2021). The tested antibiotics in this study were penicillin (10 µg), oxacillin (5µg), cefoxitin (30µg), gentamicin (10µg), tobramycin (10µg), amikacin (30µg), vancomycin (30µg), rifampin (30µg), spiramycin (50µg), lincomycin (15µg), pristinamycin (15µg), erythromycin (15 µg), chloramphenicol (30µg), imipenem (10µg), and trimethoprim/ sulfamethoxazole (25µg), cefotaxim (5µg), cefazolin (30µg), ampicillin (10µg) and amoxicillin (20µg).

Biofilm Detection Methods:

The isolates were subjected to

biofilm detection by the three phenotypic methods: tube adherence method (TAM), Congo red agar (CRA) method and tissue culture plate (TCP) method.

(a) Tube Adherence Method:

A loopful of the bacterial suspension was transferred to a tube containing 10 ml of brain heart infusion (BHI) broth (Merck, Germany) medium with glucose (2%). The tubes were incubated for 24 h at 37°C. The tube's contents were then removed and washed with Phosphate Buffered Saline PBS (pH 7.3). Then the dried tubes were stained with Crystal violet (1%). Excess dye was washed with sterile distilled water. Tubes were dried in an inverted position. The isolates forming slime or biofilm-based on Crystal violet color's thickness on the tube's bottom and the wall was observed and registered. The observation of a thick layer visible on the tube wall and bottom was considered a strong biofilm. The observation of a visible thin layer on the tube wall and bottom was considered a moderate biofilm. The lack of a visible layer on the tube wall and bottom was considered non-biofilm formation and negative. The experiment was performed in three replications (Mishra *et al.*,2015).

(b) Congo Red Agar Method

Congo Red Agar (CRA) was prepared by the method described by Freeman *et al* . (1989). After inoculating the plates with the isolates in triplicate, they were incubated at 37°C under 10% of CO_2 tension overnight. The plates were qualitatively examined for the CRA color intensity changes after incubation, where the color intensity was directly proportional to a biofilm production capacity. Black colonies with a dry crystalline consistency were considered strong biofilm formers.

Weak biofilm producers usually remained pink, though occasional darkening at the colonie's centers was observed. Darkened colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result (Sevanan *et al.,* 2011).

(c) Tissue Culture Plate Method:

Isolates obtained from fresh agar plates were inoculated in Trypticase Soy Broth and incubated for 24 h at 37°C, then diluted with fresh Trypticase Soya Broth in 1 in 100 dilutions. Individual wells of sterile polystyrene (96 well-flat bottom tissue culture plate (TCP) wells) were filled with 0.2 ml aliquots of the diluted cultures, and only broth served as a control to check sterility and the media nonspecific binding. The TCP was incubated for 18–24 h at 37°C. After incubation, the content of each well was delicately removed by tapping the plates. Then wells were washed four times with 0.2 ml of PBS (pH 7.2) to remove free-floating “planktonic” bacteria. Wells were stained with crystal violet (0.1%). The excess stain was then removed by washing with deionized water, and the plate was kept for drying (Christensen *et al.*,1985).

If the biofilm is formed by organisms, then wells are uniformly stained with crystal violet. The optical density (OD) of stained adherent bacteria was determined with a micro-ELISA auto reader at a wavelength of 570 nm (OD 570 nm).

Biofilm production was classified as negative, weak, moderate, and strong based on the cutoff value, calculated according to the following formula, using the optical density (OD) values (Shrestha *et al.*,2018):

The used criteria were as follows:

(i) $OD \leq$ OD cutoff = non-biofilm former

(ii) OD cutoff $< OD \leq 2 \times$ OD cutoff = Weak biofilm former.

(iii) $2 \times$ OD cutoff $< OD \leq 4 \times$ OD cutoff = Moderate biofilm former

(iv) $OD > 4 \times$ OD cutoff = Strong biofilm former.

RESULTS

A total of 104 samples with significant bacterial growth were collected during the study period. UC tip and CVF revealed a polymicrobial growth resulting

in a total of 80 bacteria (51 Gram-positive and 29 Gram-negative), were isolated and identified, with the most frequent being coagulase-negative *Staphylococci* (CoNS) representing 36.8% (46/80) of the total isolates.The most frequently isolated enteric bacteria were *E. coli*, *K. pneumoniae* and *Citrobacter spp.* (Table.1) presents the device-wise distribution of samples according to the corresponding type and number of isolated organisms.

Table 1: Device-wise distribution of samples and the corresponding type and number of isolated organisms.

Indwelling medical device	No. of samples	*E. coli*	*K. pneumoniae*	*Enterobacter spp*	*Shigella spp*	*Citrobacter spp.*	*P. aeruginosa*	*S. epidermidis*	*S. aureus*	Total
UC tip	16	4	2	3	2	3	3	5	1	23
CVF tip	11	3					1	9	2	15
ET tube	3						1	2		3
CVJ tip	1							1		1
PV tip	73		2			1	4	29	2	38
Total	104	7	4	3	2	4	9	46	5	80

E. coli : Escherichia coli, K. pneumoniae :Klebsiella pneumoniae, P. aeruginosa :Pseudomonas aeruginosa, S. epidermidis : Staphylococcus epidermidis, S. aureus : Staphylococcus aureus .

TCP method detected 68 (85%) of biofilm producers and 12 (15%) of non-biofilm producers. Biofilm-producing Gram-negative organisms (n = 22) were enteric bacteria (55.17 %) and *P. aeruginosa* (20.68%); for biofilm-producing Gram-positive organisms (n=46) were *S. epidermidis* (82.35%) and *Staphylococcus aureus* (7.84 %). *S. epidermidis* was found to be the most frequent biofilm producer by the TCP method (Table.2, Fig.1, Fig..2 and Fig. 3).

Similarly, the tube method showed 67 (83.75%) of biofilm producers and 13 (16.25%) of non-biofilm producers. Strong biofilm production was caused by *P. aeruginosa*. By CRA method, 57 (71.25%) isolates were considered as biofilm producers and 23 (28.75 %) as non-biofilm producers.

Table 2: Biofilm production by the three used methods

		CRA		TAM		TCP	
		+	-	+	-	+	-
Gram-negative	29	15	14	24	5	22 (75.86%)	7
Enterobacterial	20	11	9	15	5	16 (55.17 %)	4
P. aeruginosa	9	4	5	9	0	6 (20.68 %)	3
Gram-positive	51	42	9	43	8	46 (90.19%)	5
S. aureus	5	3	2	4	1	4 (7.84 %)	1
S. epidermidis	46	39	7	39	7	42 (82.35 %)	4
Total	80	57	23	67	13	68 (85%)	12(15%)

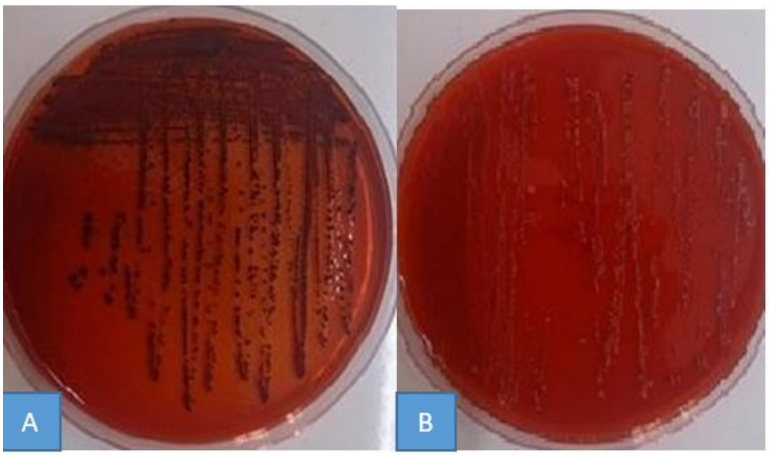

Fig .1: Slime production by the strains isolated on Congo red medium
A: Slime producing, **B:** Non-slime producing

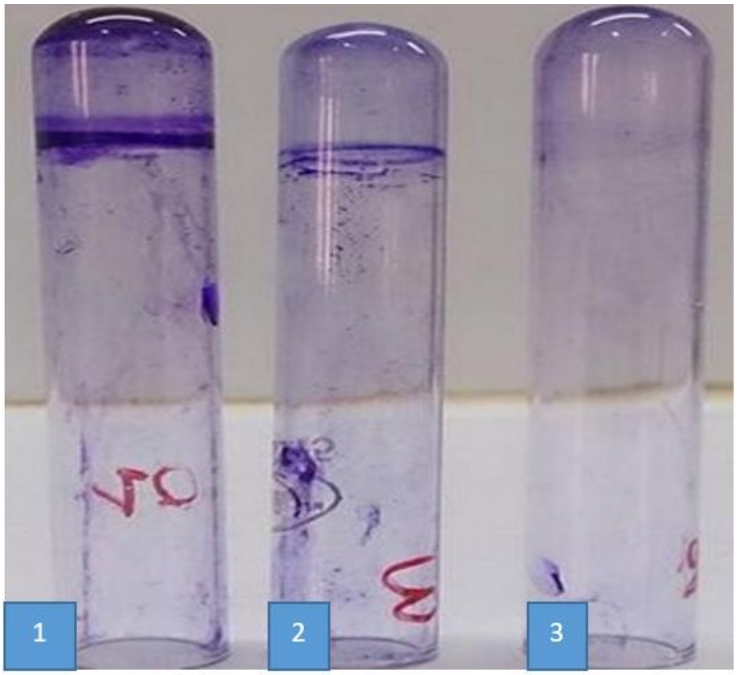

Fig.2: Tube Adherence method.
1: Strongly adherent, **2:** Moderately adherent and **3:** Non-adherent

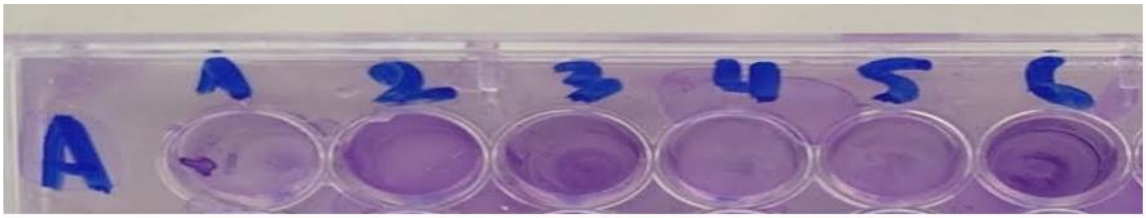

Fig.3: Tissue culture plate method for biofilm detection

This antibiotic susceptibility study of the strains isolated at Mohamed Boudiaf Hospital revealed of Ouargla (Algeria) revealed a significant level of resistance against several antibiotics; we have observed a higher antibiotic resistance in biofilm-producing bacteria than in non-biofilm producers. By the standard method (TCP). (Table.3) illustrates the resistant pattern of the Gram-negative bacterial

isolates, showing a high resistance rate (>80%) to Amoxicillin, Ampicillin, Cefazolin, Cefoxitin and Cefotaxime, with 4 isolates resistant to imipenem. These strains were also resistant to aminosides with rates ranging from 36.36 % for Amikacin to 50 % for Gentamycin. Resistance rates of isolates to Trimethoprim and Cloramphénicol were 36.36 % and 63.63 % respectively. In contrast to Gram-negative bacteria, Gram-positive bacteria isolates showed a higher level of resistance to all antibiotics, but to some were sensitive to Pristinamycin and Spiramycin. Eleven of the *Staphylocooccus* strains were resistant to vancomycin. (Table.4) illustrates the resistance pattern of the Gram-positive isolates. For all others, antibiotic resistance associated with biofilm producers was greater than that with biofilm non-producers.

Table 3: Antibiotic resistance pattern of Gram-negative isolates

Antibiotics	Biofilm positive isolates (n = 22)	%	Biofilm negative isolates (n = 7)	%
Amoxicillin (AML)	21	95.45	5	71.42
Ampicillin (AMP)	21	95.45	5	71.42
Cefazolin (CZ)	19	86.36	6	85.71
Cefoxitin (FOX)	19	86.36	5	71.42
Cefotaxime (CTX)	21	95.45	6	85.71
Imipenem (IMP)	4	18.18	1	14.28
Amikacin (AN)	8	36.36	2	28.57
Gentamycin (CN)	11	50.00	3	42.85
Trimethoprim (SXT)	18	36.36	2	28.57
Chloramphenicol (C)	14	63.63	3	42.85

Table 4: Antibiotic resistance pattern of Gram-positive isolates

Antibiotics	Biofilm positive isolates (n = 46)	%	Biofilm negative isolates (n = 5)	%
Pénicillin (P)	45	97.82	2	40
Oxacillin (OX)	40	86.95	4	80
Tobramycin (TOB)	36	78.26	3	60
Gentamycin (CN)	28	60.86	2	40
Erythromycin (E)	31	67.39	2	40
Lincomycin (L)	24	52.17	2	40
Pristinamycin (PM)	21	45.65	2	40
Vancomycin (VA)	11	23.91	1	20
Rifampycin (RIF)	25	54.34	2	40
Spiramycin (SP)	16	34.78	1	20

DISCUSSION

Biofilm-producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. Despite the different antimicrobial therapies available, the management of bacterial infections caused by biofilms remains problematic (Schierle *et al.*,2009).

To our knowledge, this is the first and only study on the microbiological characteristics of MD infections in southern Algeria. Although our data reflect the MD epidemiology infections in the region, they could be of great interest and provide useful information for MD

infections management in other regions. In the present study, we detected the *in-vitro* biofilm-forming capacity of bacteria isolated from indwelling medical devices of hospitalized patients and their association with antimicrobial resistance.

A total of 80 bacteria were isolated and identified from the MDs; 63.75 % of Gram-positive bacteria were isolated, which were mainly CoNS; with a predominance of *S. epidermidis*. These results closely agree with those reported by Percival *et al.* (2015); who estimated that around 80% of the bacteria implicated in infections associated with medical devices are *S. epidermidis*.

In several studies, *S. epidermidis* was the most frequently isolated CoNS species and constitutes a significant part of the normal bacterial flora of human skin and mucous membranes from where it is easily introduced as a contaminant during surgical implantation of the polymer device (Otto, 2008).

Gram-negative bacteria constituted 36.25 % of the isolated bacteria, with a high frequency of enteric bacteria such as *E. coli, K. pneumonia, Enterobacter spp, Shigella spp, Citrobacter spp.* and *Pseudomonas aeruginosa*. (Almalki and Varghese,2020) detected 89% of Gram-negative bacteria on the same devices, of which *E. coli* was the most frequently detected (26%) (Almalki and Varghese,2020).

In this study, the detection and comparison of biofilm-forming capacity were performed using three *in-vitro* methods, TCP, TM and CRA, respectively. TCP has been used as a gold standard method, while TM and CRA are used as screening tests (Mishra et *al.*, 2015). The TCP method, which is the most widely used technique, was considered the 'gold standard test for sensitive, accurate and reproducible screening method for the detection of biofilm production in clinical isolates (Oli *et al.*, 2012).

There was a good association between the TAM and the TCP method, then between the CRA method and the TCP method in this study. In a similar study, the TCP method revealed 64.7 % of biofilm producers and 36.3 % of non - biofilm producers (Hassan *et al.,* 2011 a). They also showed a good correlation between the TAM and the TCP method, but a very low correlation between the CRA and the TCP methods. Moreover, the TM is cost-effective and widely available, which should be kept into consideration in a developing country as Algeria. Therefore, the TM may be used for the screening of biofilms during routine laboratory work at the hospital. Biofilm formation depends on many factors such as environment, nutrient availability, geographical origin, specimen types, surface adhesion characteristics and genetic composition of the organism.

These factors may have affected the data and contributed to the high prevalence observed in the present study. However, it is not known how these factors are involved. Biofilms can form on any wound when planktonic bacteria are not eliminated by the host's immune system or by exogenous antimicrobial agents (Hurlow *et al.*,2015). Biofilm infections are clinically important because, the bacteria in biofilms present recalcitrance to antimicrobial compounds (Hassan *et al.,* 2011 b). Biofilm-forming bacteria are therefore a public health challenge for those requiring indwelling medical devices (Vuong and Otto,2002).

The antibiotic resistance was highly alarming in the clinical isolates obtained from patients with DRI (device-related infections) in our institution. Resistance was found to all the controlled antibiotics, *Staphylococcus* was an important etiological agent of DRI, and a variety of antibiotic-resistant mechanisms were investigated in biofilm isolates (Grinholc *et al.*,2007). CoNS may adhere directly to the device plastic polymers via fimbria-like surface protein structures or via a capsular polysaccharidic adhesin (Veenstra *et al.*,1996).

A significant correlation was detected between the antibiotic resistance exposure and the biofilm-production capacity, particularly of Gram-negative bacteria, in our hospital. The high resistance and biofilm production among the DRI isolates, in our study, indicated that the bacteria in the biofilms showed an altered character, and the high resistance could be attributed to the isolates' biofilm production capacity.

The higher antibiotic resistance pattern showed by biofilm-producing bacteria than biofilm non-producers, could be due to restricted and limited antibiotics penetration into biofilms, the bacteria decreased growth rate in biofilm, the high expression of efflux pump and expression and exchange of resistance genes among bacteria within a biofilm. To eradicate the biofilm producers, high antimicrobials concentrations may be necessary and required. However, this may not always be practical *in-vivo* due to the toxicity risk and associated side effects. Therefore, low-concentration combination therapies may be effective to eradicate biofilm-related *Staphylococcal* infections, including those by MRSA *(Methicillin-resistant Staphylococcus aureus*) (Wu *et al.*,2013). The early biofilm producers detection and screening followed by their antimicrobial susceptibility tests is important for the appropriate antimicrobial agent selection.

A study by Qu *et al.* (2010) reported that Biofilm communities of Coagulase-negative *Staphylococci* could not be eradicated with higher pharmacological concentrations of gentamicin, oxacillin and vancomycin. A higher amount of antibiotics is needed to control the *Staphylococcus aureus* biofilm than the planktonic organism. Saginur *et al.* (2005) and Japoni *et al.* (2004) also concluded similarly to our study result.

The wide difference in resistance rates associated with DRI may be attributed to the lack of infection control programs as well as the injudicious and inappropriate use of antibiotics in our country. The bacteria nature complex is altered due to such widespread misuse and overuse of antibiotics in our country, In addition, the biofilm-producing ability of the bacteria further complicates the resistance problem. The tube method can be an effective alternative to sophisticated microscopy techniques for screening the biofilm-producing ability of bacteria, in resource-limited countries. The device-related infections and associated antibiotic resistance by biofilm-producing isolates are now emerging problems. The varying resistance pattern of organisms isolated in our institution focuses on the importance of studying the infection pattern in all environments and by providing the antibiotics instructions in the control of these infections.

CONCLUSION

The clinical isolates recovered from IMDs of hospitalized patients show a high degree of biofilm formation. A higher rate of antimicrobial resistance is demonstrated by biofilm producers than by non-biofilm producers. We can conclude from our study that the TCP is a quantitative and reliable method for microorganisms biofilm-forming detection. When compared to the TM and the CRA methods, and the TCP can be recommended as a general screening method for the detection of biofilm-producing bacteria in the laboratory.

Therefore, we recommend regular control of biofilm formation in IMDs bacterial isolates; and their antimicrobial resistance profiles. This may help us to formulate an effective antimicrobial strategy for the early treatment of IMDs infection.

Conflicts of Interest

The authors declare that they have no conflicts of interest with respect to the publication of this document.

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